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ACTIVATION OF PRORENNIN

by

Nazar A. Shukri

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Food Science and Technology

UTAH STATE UNIVERSITY »
Logan, Utah

1969

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Nazar A. Shukri

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ABSTRACT

Activation of Prorennin

by

Nazar A. Shukri, Doctor of Philosophy

Utah State University, 1969

Major Professor: Dr. C. A. Ernstrom
Department: Food Science and Technology

Purified prorennin appeared to be homogeneous when subjected to chromatography on a Diethylaminoethyl-cellulose and to electrophoreses in starch-urea-gel. Crystalline rennin was heterogeneous, and was resolved into six components when analyzed electrophoretically. When crystalline rennin was chromatographed on a column of Diethylaminoethyl-cellulose two distinguishable peaks were observed which corresponded to B- and C-rennin. Unlike crystalline rennin, rennin freshly activated at pH 2.0 or 5.0 was essentially homogeneous.

Rennin resulting from activation at pH 2.0 and 5.0 appeared to be different. Rennin activated at pH 5.0 was eluted faster from Diethylaminoethyl-cellulose column, and moved slightly faster in starch-urea-gel electrophoresis, than rennin activated at pH 2.0. Amino acid analysis showed that rennin activated at pH 2.0 had more arginine and less proline than that activated at pH 5.0. This could be accounted for the differences in chromatographic

and electrophoretic behavior of these rennins.

Crystalline rennin exhibited more resistance to urea denaturation than prorennin. This suggested a fundamental difference in their secondary or (and) tertiary structures. It also showed the importance of intramolecular H-bonding for enzyme activity. An increase in crystalline rennin components was accompanied its prolonged exposure to 6 M urea. This was not the case with prorennin.

(77 pages)

INTRODUCTION

Rennin is the main milk-clotting enzyme obtained from the fourth stomach of young calves. It is secreted as an inactive precursor called prorennin, which is transformed into an active enzyme in the environment of the abomasum. Rennin is a proteolytic enzyme, which exhibits its maximum activity on hemoglobin, bovine serum albumin, and casein in the region of pH 3.0-4.0 (3, 6, 10, 13). It has a specificity similar to, but narrower than, that of pepsin (10). Rennin has been crystallized (2, 6, 9, 21), but the crystalline enzyme was found to be heterogeneous (9). Prorennin has not been crystallized, and has no proteolytic activity. Conflicting reports have been published concerning its homogeneity. Foltman (16) reported the heterogeneity of prorennin, while Bundy et al. (5) and Djurtoft et al. (7) claimed that it was homogeneous. Prorennin can be activated in vitro at pH 5.0 (33, 37). However, as the pH is decreased the rate of activation increases markedly (27, 33).

Rennin has been used in cheese making for at least twenty centuries. Recently a rennet shortage has developed in the market due to a decreased dairy-cow population, increased cheese production, and an increasing tendency to raise calves for beef rather than for veal. This rennet shortage, which shows no sign of relaxing in the near future, makes it urgent to learn as much as possible about rennin and its function.

This research was originated to study the kinetics of activation of prorennin at low pH values (37). When it appeared that activation at pH 5.0 and at

pH 2.0 were different, it suggested the possibility of different pathways of activation, and raised questions about the structural similarity of rennin activated under these two conditions.

This study was undertaken to see if there were differences between rennin activated at pH 2.0 and that activated by autocatalysis at pH 5.0.

Specifically, the objectives of this work were to determine;

1. Whether or not there is a difference between rennin activated at pH 2.0 and that activated at pH 5.0.
2. To consider and clarify the contradiction concerning prorennin homogeneity.

LITERATURE REVIEW

Enzymes are synthesized in living systems either as complete active catalysts or as inactive precursors which are activated outside the secreting cells. Rennin falls in the second category. It is secreted in an inactive form called prorennin, and is activated in the environment of the stomach. This activation may also be brought about in vitro.

Prorennin. The existence of prorennin was first recognized by Hammarsten (20). However, Kleiner and Tauber (26, 27) were the first to partially isolate this zymogen and study its properties. They reported that the rate of conversion of prorennin to rennin increased with increasing acidity down to pH 1.0. They also demonstrated that prorennin was stable in the region of pH 9.0-10.0 where rennin was rapidly inactivated.

Berridge (42) observed some proteolytic features involved in the activation of prorennin, and pointed out the need for pure prorennin before definitive studies could be made.

Foltmann (12) devised a method for purifying prorennin by employing salt extraction of dried calf stomachs. He was able to prepare material in which 50% of the protein was prorennin. He reported that the isoelectric point of prorennin was pH 5.0 or slightly lower as determined by paper electrophoresis. Foltman (15, 16) further purified prorennin by ion-exchange chromatography on a column of Diethylaminoethyl-cellulose (DEAE-cellulose), and found his preparation

to be heterogeneous and capable of being fractionated into at least two components. He designated the main fraction prorennin-B, and the minor one prorennin-A. Upon activation, each of these fractions gave rise to different rennin components. He showed that activation at pH 4.7 was partially autocatalytic, while that at pH 2.0 resembled a second-order reaction (18). His work also showed that activation of prorennin was brought about by hydrolysis of peptide material from the N-terminal end of the prorennin molecule. Applying Sanger's (35) method for determining the N-terminal amino acid, Foltmann found that alanine occupied the N-terminal end of the prorennin molecule. In a later work (17) he showed that during activation at pH 2.0 and 25 C, three peptides were split from the N-terminal end of the prorennin molecule. He reported the amino acid composition for these peptides along with those for rennin and prorennin (Table 1). He found that the amino acid composition of prorennin-B was similar to that of total prorennin. Also, chromatographically purified B-rennin resulting from the activation of prorennin-B, had almost the same amino acid composition as rennin. He was able to account for all the amino acids in prorennin from the amino acid composition of rennin and the peptides released during activation. Foltmann also found that in spite of the homogeneity of prorennin-B, the enzyme derived from it was heterogeneous. He suggested that the activation process might take different pathways leading to different enzyme fractions.

Bundy et al. (5) described another method for purifying prorennin. The method consisted of twice extracting calf stomachs with acetone at -5 C. The acetone-extracted material was dried and re-extracted with tris buffer at

pH 7.2. Ammonium sulfate was then added to precipitate the protein. The prorennin was further purified by chromatography on a column of DEAE-cellulose and rechromatography on a column of DEAE-Sephadex A-50 medium. They concluded that prorennin was homogeneous as indicated by sedimentation analysis and column chromatography. This was in contrast to the findings of Foltmann (16). They also showed that activation of prorennin at pH 4.4 was autocatalytic, and that the release of peptide material occurred during the activation process.

Rand and Ernstrom (33), studied the activation of prorennin between pH 2.0 and 5.5. They demonstrated that activation at pH 4.7 and 5.0 was predominantly autocatalytic. Below pH 4.0, activation was too rapid, even at low temperatures, to follow accurately, while that at pH 5.5 and above was extremely slow. Sodium chloride at a concentration of 1.5-2.0 M markedly enhanced the rate of activation of prorennin at pH 5.0, but when the sodium chloride was increased to 2.6 M, it was detrimental both to the activation rate and the yield of enzyme. Activation at pH 2.0 (25 C) in the presence of sodium chloride, even at a concentration of 0.05 M, reduced the yield of active enzyme. This reduction was more pronounced as the salt (NaCl) concentration was increased. The highest amount of active rennin was obtained from activation at pH 5.0 in 1.5 M sodium chloride. The amount formed at pH 5.0 was enough greater than that formed at pH 4.7 to be economically important. In a later report (34) Rand and Ernstrom showed that activation of prorennin could be catalyzed by pepsin. The pepsin-catalyzed activation at pH 5.5 and 6.0, proceeded rapidly at first

and resembled a zero-order reaction. As the activation proceeded it changed rather abruptly to a much slower rate.

Shukri (37) found that glycerol, in activation mixtures, had the ability to reduce activation rates without significantly affecting the nature of the reaction or the final yield of active enzyme. This finding made it possible to accurately study the activation of prorennin at low pH values. He showed that activation of prorennin at pH values below 4.0 followed first-order kinetics. He also verified the predominantly autocatalytic nature of activation above pH 4.0.

Djurtoft et al. (7) determined the molecular weight of prorennin by sedimentation-diffusion and by gel filtration on a column of Sephadex G-100. They reported a molecular weight of approximately 36,000, and found that a small amount of the prorennin had an elution volume which corresponded to a molecular weight of about 70,000. Foltmann (18) suggested that some of the prorennin was present as dimers. The most interesting point in this study was that these workers who used gel filtration and sedimentation-diffusion did not observe heterogeneity comparable to that observed by column chromatography (16). Based on the sedimentation analysis and N-terminal amino acid data, Bundy et al. (5) estimated the molecular weight of prorennin to be 40,000-50,000. They also observed that the sedimentation constant increased with prorennin concentration, which suggested a concentration-dependent association of prorennin molecules.

Rennin. Rennin has been crystallized by Berridge (2), Hankinson (21), De Baun et al. (6), and Ernstrom (9). The latter showed that crystalline rennin

was heterogeneous and contained three components as shown by free-boundary electrophoresis. This finding was confirmed by Foltmann (14), when he resolved crystalline rennin into three active and one inactive component on a chromatographic column of DEAE-cellulose. He designated the active fractions as A-, B-, and C-rennin in decreasing order of their specific activities. Jirgensons et al. (25) showed that a chromatographically pure rennin could be obtained by fractionation on a DEAE chromatography column. They reported that the N-terminal and C-terminal amino acids on rennin were glycine and leucine or isoleucine respectively. They also reported that rennin belonged to a class of abnormal proteins which did not have a helical configuration. They based this conclusion on the fact that rennin behaved abnormally with respect to optical rotatory dispersion.

Mickelsen and Ernstrom (28) investigated the stability of rennin between pH 2.5 and 6.8 in buffers of glycine-HCl (pH 2.5-3.0), lactic acid-sodium lactate (3.0-4.0), acetic acid-sodium acetate (4.0-5.2), cacodylic acid-cacodylate (5.2-6.0), and phosphate (6.0-6.8) at 0.03 ionic strength. They reported that rennin had its maximum stability between pH 5.0-6.0. In the region of pH 3.0-4.9 rennin was less stable and had an instability maximum at pH 3.8. When the ionic strength was increased from 0.03 to 1.0 with sodium chloride or potassium chloride it was extremely damaging to rennin at pH 3.8. In examining the stability of the enzyme at this pH in the presence of different anions and cations, they found that chloride was responsible for the pronounced deterioration of the enzyme. When lactate was the only salt present, an increase in ionic strength from 0.03 to 1.0 actually reduced activity losses by

50%.

Foltmann (13) examined the stability of solutions of crystalline rennin between pH 2.0 and 7.0 in buffers of citrate (2.0-4.0), acetate (4.0-5.1), and phosphate (pH 5.1-7.0). The ionic strength of all buffers was 0.05. He demonstrated that rennin had maximum stability at pH 5.5-6.0, with moderate stability at pH 2.0. He also showed that rennin was unstable between pH 3.0 and 4.0, and above 6.5. At pH 4.0, citrate buffer appeared to cause slightly more loss than acetate buffer, however, acetate and phosphate buffers had identical effects at pH 5.1.

Schwander et al. (36) reported the molecular weight of rennin to be 40,000 as determined by sedimentation and diffusion analysis. Gel filtration and sedimentation-diffusion studies by Djurtoft et al. (7) showed that rennin had a molecular weight of about 33,000. In the gel filtration experiment however, a small amount of rennin had an elution volume which corresponded to a molecular weight of 70,000, indicating that rennin, like prorennin, must form dimers in solutions. Association between rennin molecules was observed earlier by Baldwin and Wake (1) in their sedimentation studies on rennin. They showed that the degree of association depended on both the ionic strength and enzyme concentration. At 0.02 ionic strength, the Schlieren patterns showed two partly resolved peaks at high rennin concentration. When the concentration was reduced they observed only one peak. When the ionic strength was increased to 0.32 the patterns showed only one peak at all rennin concentrations.

Hill and Laing (23) showed that rennin became inactivated when

photo-oxidized with methylene blue at pH 6.4. During photo-oxidation 14-16 moles of O_2 were absorbed by one mole of enzyme. Under these conditions 60% of the histidine residues were destroyed. Modification of other photo-oxidizable side chains (tryptophan and methionine) by specific treatments had no effect on activity. From this they concluded that one or more histidine side chains form part of the active center of rennin.

Table 1. Amino acid composition of prorennin-B, total prorennin, B-rennin, and total rennin (Moles/ 10^5 grams of protein) (17, p. 280-281)

Amino acid	Prorennin-B	Total prorennin	B-rennin	Total rennin
Lysine	35.8	35.3	26.5	24.0
Histidine	13.8	13.9	13.4	14.1
NH ₃		114.8		113.3
Arginine	19.4	18.5	15.8	16.7
Aspartic	92.2	92.7	97.7	103.3
Threonine	56.4	57.2	61.0	61.8
Serine	84.4	80.6	86.9	80.1
Glutamic	100.3	101.5	94.3	93.3
Proline	38.6	41.0	39.2	43.7
Glycine	78.7	77.7	79.8	75.4
Alanine	41.5	41.7	41.8	41.1
Cystine 1/2	14.0	13.3	15.9	12.3
Valine	63.0	57.5	68.1	66.3
Methionine	18.7	18.9	21.7	21.4
Isoleucine	52.6	50.5	49.4	50.6
Leucine	70.6	70.5	60.4	64.0
Tyrosine	50.7	52.2	50.1	51.8
Phenylalanine	45.5	45.2	44.9	45.0
Tryptophan	11.2	11.2	13.6	12.4
Amide	94	94	100	100

EXPERIMENTAL METHODS

Determination of milk-clotting activity. Enzyme activity was measured on a substrate suggested by Berridge (4), using a method described by Ernstrom (9). The substrate was made by dissolving 120 g low-heat nonfat dry milk in one liter of 0.01 M calcium chloride and holding for 20 hours at 4 C. Twenty-five ml samples from the bulk substrate were placed in 125-ml wide-mouth bottles, and tempered to 30 C in a water bath. One ml of the properly diluted enzyme solution was added to each bottle, and the clotting time measured and recorded. Activities of unknown enzyme solutions were compared with the activity of a standard rennet extract which was maintained at a constant activity throughout this study. The standard rennet extract, arbitrarily given a value of 100 RU/ml, had activity such that one ml of a 1:250 dilution produced visible clotting of the substrate in 125 ± 5 counts on a test apparatus (40) rotating at 20 counts per minute. Variations in the response of different lots of substrate to rennin accounted for some variation in clotting time. Consequently, whenever rennin solutions were tested, the activity of the standard was measured simultaneously on the same substrate.

Purification of rennin. Rennin was purified by a method described by Ernstrom (9). A concentrated rennet extract (double strength, about 170 RU/ml) was adjusted to pH 5.0 with 3N HCl and saturated with sodium chloride. The precipitate which formed was separated by centrifuge for 45 min. at 5000 g.

It was then redissolved in one-half the original volume of water and adjusted to pH 6.3 with dilute sodium hydroxide. These steps were repeated until four salt precipitates had been obtained. The fourth precipitate was also redissolved in water and the pH of the solution adjusted to pH 6.3 with dilute sodium hydroxide. Potassium alum, dissolved in a small amount of water, was added at the rate of 10 g per liter. The solution was immediately neutralized to pH 6.3 with dilute sodium hydroxide. The aluminum hydroxide gel was removed by centrifuging. The supernatant was adjusted to pH 4.6 with dilute hydrochloric acid and the rennin again precipitated by saturating with sodium chloride. The precipitated rennin, which was obtained by centrifugation, was dissolved in a minimum of 0.05 M phosphate buffer at pH 6.8 and dialyzed against salting-in buffer (50 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ plus 15 g CH_3COONa , $3\text{H}_2\text{O}$ per liter, adjusted to pH 5.4 with 10 N H_2SO_4) (3). Crystals which formed during dialysis were centrifuged and washed twice in salting-in-buffer and twice at pH 5.6 in 0.05 M acetate buffer containing 0.3% sodium chloride. Finally, the rennin was redissolved in a small amount of 0.05 M phosphate buffer (pH 6.8), dialyzed against water, and lyophilized. The final product had a specific activity of 380 RU/mg nitrogen. The highest reported specific activity for crystalline rennin is 400 RU/mg nitrogen (9).

Purification of prorennin. Partially purified prorennin was prepared by a modification of Foltmann's method (12, 15, 16). Unsalted dry-blown calves' stomachs were extracted at 3 C in a 10% sodium chloride solution (pH 8.0) containing 0.1 M sodium borate. The extract was then adjusted to

pH 7.0 with 3N HCl. Potassium alum (10 grams per liter of extract) dissolved in small amount of water was added to the extract and the acidic salt immediately neutralized to pH 6.3 with saturated disodium phosphate. The gel which formed was removed by centrifuging. The supernatant was adjusted to pH 8.0 with 3N NaOH, saturated with sodium chloride, and left overnight at 3 C. The pH was adjusted to 7.0 with 3N HCl, and the enzyme was separated by centrifuging at 5000 g for 2 hr. at 15 C. The precipitate was dissolved in a minimum of 0.1 M borate buffer (pH 8.0), and filtered. Precipitation of the enzyme was repeated once more, and the precipitate redissolved in 0.1 M borate buffer (pH 8.0). Potassium alum (5 grams per liter) was again added and the solution neutralized immediately to pH 6.3 with saturated disodium phosphate. The aluminum phosphate gel was removed by centrifuging and discarded. The supernatant was dialyzed against 0.04 M borate buffer (pH 8.0), then freeze dried.

The partially purified prorennin was further purified by ion-exchange chromatography on a column of DEAE-cellulose as recommended by Peterson and Sober (30). The partially purified prorennin was dissolved in 0.05 M phosphate buffer at pH 5.95 and applied to a preparative DEAE-cellulose column. After the sample had been drawn into the column, it was rinsed with few ml of equilibrating buffer (0.05 M phosphate, pH 5.95) and allowed to drain. The column was then connected to the reservoir which contained equilibrating buffer. After 680 ml had been eluted, the concentration of the buffer on top of the resin and in the reservoir was changed to 0.1 M (pH 5.85). Subsequently,

and in the same manner, the buffer was changed to 0.2, 0.25, and 0.3 M after 490, 840, and 465 ml respectively.

It was noted that satisfactory resolution could be achieved with a simpler elution. Hence, in later preparative experiments 0.1 M phosphate buffer at pH 5.85 was used for equilibrating the sample and starting the elution. The buffer was then changed to concentrations of 0.2 and 0.3 M after 640 and 700 ml respectively. The flow rate was about 30 ml/hr., and about 20-ml fractions were collected. Protein concentrations were determined spectrophotometrically at 280 m μ with the aid of a Ziess PMQ II spectrophotometer. Selected samples were used for activity determinations.

Buffers used in these experiments were all prepared by suitable dilution of 1.0 M phosphate buffer made by dissolving 12.92 g Na₂HPO₄ plus 125.46 g NaH₂PO₄·H₂O in a final volume of one liter. Changes in pH with concentration, were due to varying ionic strengths.

After each run fractions representing prorennin were pooled, dialyzed against distilled water (8-10 hours), and lyophilized. When prorennin solutions were concentrated by perfusion in dialysis casings prior to freeze drying, there was about 5% loss in activity accompanied by slight heterogeneity in the dried material. Therefore, concentration by this method was avoided throughout this study.

Further purification of prorennin was attempted by rechromatography with gradient elution. Once-chromatographed prorennin was dissolved in 0.1 M phosphate buffer at pH 5.85, and applied to a new preparative DEAE-cellulose

column (2.5 x 25.0 cm). After the sample had been drawn into the column, it was rinsed in with few milliliters of the equilibrating buffer (0.1 M phosphate, pH 5.85). The column was then connected to the reservoir which contained 800 ml of equilibrating buffer, and into which was allowed to flow 0.3 M phosphate buffer (pH 5.60) at the same rate as buffer moved from the reservoir onto the column. Flow rate was 25 ml/hr., and 18-ml fractions were collected. Protein concentration and activity determinations were made as previously described.

The final product used in this study had a specific activity of 320-340 prorennin units per mg nitrogen and only 0.11-0.36% of the total activity was due to active rennin. This potential activity corresponded to 80-85% of the highest reported specific activity for crystalline rennin (9).

Preparation of DEAE-cellulose column. DEAE-cellulose powder was suspended in buffer, and allowed to equilibrate and settle overnight in the refrigerator. The entire slurry was poured onto Whatman No. 1 filter paper which allowed the fines to pass through. The DEAE-cellulose was resuspended in the same buffer, and poured as a slurry into a 60 x 1.1 cm glass tube, the outlet of which was closed. When the settled resin was about 4-5 cm deep the outlet was opened and packing continued until all the slurry was poured into the column. After gravity settling was complete a pressure of 6 psi was applied to help pack the column until a constant height was achieved. The column was then transferred to a cold room at 3°C, and again washed overnight with equilibrating buffer. The column was now ready to use. After each run the column was washed with a solution containing 0.5 M NaCl and 0.05 M NaOH, rinsed with

distilled water, and equilibrated with the buffer. When the column was not in use, the liquid was kept at a height of 1 cm above the resin.

Several DEAE-cellulose columns (different sizes) were used during this study, but all were prepared and treated as described.

Activation of prorennin and resolution of activation mixtures. Activation of prorennin samples at pH 2.0 was carried out in 0.02 M phosphate buffer. Purified prorennin powder was dissolved in the phosphate buffer at a concentration of 3-4 mg/ml. Samples remained at the activation pH for 20 min. before neutralization with saturated Na_2HPO_4 to pH 6.0 ± 0.1 . The samples were then dialyzed against 0.002 M cacodylate buffer at pH 6.0 (6-8 hours), and applied to the DEAE-cellulose column. When the size of the dialyzed samples was large, they were freeze dried first and then dissolved in a minimum amount of buffer before applying to the column.

It was noticed that a fine white nondialyzable precipitate formed during neutralization. Hence, this precipitate was removed by centrifugation before the sample was applied to the column.

After the sample had been drawn into the column and rinsed with a few milliliters of the equilibrating buffer (0.02 M phosphate buffer at pH 6.0), the column was connected to the reservoir for gradient elution. The reservoir contained 400 ml equilibrating buffer and into it was allowed to flow a 0.4 M phosphate buffer at pH 5.5.

The column was operated at 3 C, and the resin volume was 38 x 1.1 cm. The flow rate was 15 ml/hr., and the fraction size was 5.0 ml. Fractions

were collected from the column and the protein concentration determined spectrophotometrically at 280 m μ with a Ziess PMQ II spectrophotometer. Selected samples were used for determination of enzyme activity and phosphorous concentration.

Prorennin samples were activated at pH 5.0 in 0.3 M acetate buffer. Activation was allowed to proceed to completion whereupon samples were neutralized to pH 6.0 ± 0.1 and treated as those activated at pH 2.0.

It is noteworthy that upon neutralization of samples activated at pH 5.0, there was no precipitate like that which resulted from neutralizing mixtures activated at pH 2.0.

Activation mixtures were prepared for electrophoresis in a similar way except the prorennin concentration was 1.7 mg/ml, and the dialyzed and neutralized activation mixtures were lyophilized.

Starch-urea-gel electrophoresis. Starch-urea-gel electrophoresis was carried out by a modification of the procedure described by El-Negoumy (8). The gel was made by mixing 27 g hydrolyzed starch (Connaught Medical Laboratories, Toronto, Canada) with 150 ml 0.015 M tris buffer adjusted to pH 8.3 with citric acid. The mixture was heated to boiling over a direct flame while being shaken by hand. It was then removed from the flame and 55 g of urea was added. Heating was resumed to a second boiling after which the hot gel was allowed to cool to about 65 C. Vacuum was then applied from a water aspirator to remove the air bubbles. A relief valve in the vacuum line was manually operated to prevent the liquid from being drawn into the line. One ml 2-mercapto-ethanol was then added with gentle mixing (29, 39). The warm gel was poured

into a plexiglass frame (inside dimensions 0.3 x 13.8 x 22.2 cm) and covered with a piece of glass. The excess gel was forced out and a heavy object was placed on the glass plate. The gel was aged for 16-20 hours at room temperature.

The conductivity buffer for electrophoresis was a modification of Poulik's buffer (31). Two one-liter glass loaf pans were used as electrode vessels into each of which was placed 750 ml 0.25 M borate conductivity buffer at pH 8.3. Bridges between the buffer and the gel were made of 8 sheets of Whatman No. 1 filter paper. The conductivity buffer was used for a maximum of ten days before replacing with fresh buffer.

After aging, a cut was made across the gel about 10 cm from the end. Samples containing 1% protein in 6 M urea, were absorbed onto inserts made from S & S No. 740-E absorbent paper. These were then blotted on tissue paper and placed in the cut in the gel. The gel was then covered with a sheet of Saran wrap to prevent drying.

Electrophoresis was carried out at room temperature. A voltage of 175 V with a current of 12 ma, was applied for 15-17 min. The power was then turned off and the inserts removed. Electrophoresis was then resumed under the same conditions and carried on for 4-4 1/2 hours. At the end of the run the gel was stained for 20 min., with a saturated solution of Amido black in a solvent consisting of methanol, water, and acetic acid (5:5:1) (38). The excess dye was washed off and the gel destained in 3-4 changes of the solvent.

Activation mixtures were prepared by activating samples of prorennin and neutralizing and dialyzing them as already explained. Samples were then

freeze dried and the powder kept in the refrigerator. A 1% solution of the dried samples in 6 M urea was made and applied to the starch-urea-gel. Along with these, samples of prorennin, rennin, and pepsin (3 x crystallized) were run for identification and comparison. Activity tests were not run on fractions separated by electrophoresis because of the denaturing effect of urea.

Amino acid analysis.¹ Protein samples were dried over P_2O_5 for three days after which they were weighed and placed into 100-ml round bottom flasks. Fifty ml 6N HCl was added to each flask and the solutions were purged with high purity dry nitrogen for about 5 min. The flasks were then closed with a teflon stopper. After evacuation, the samples were autoclaved at 110 C for 22 hours. The standard method for amino acid analysis as described by Spackman et al. (41) was followed using a Beckman amino acid analyzer.

Nitrogen determination. The nitrogen content of rennin and prorennin samples was measured by a semimicro Kjeldahl Method (24).

Phosphorus determination. The inorganic phosphorus content of eluted fractions was estimated by the method of Fiske and Subbarow (11).

¹Amino acid analyses were performed by Dr. M. A. Stahmann, Dept. of Biochemistry, University of Wisconsin, Madison, Wisconsin.

RESULTS

Purification and homogeneity of prorennin. A typical fractionation of partially purified prorennin by stepwise elution from a column of DEAE-cellulose is shown in Figure 1. Peak I consisted of impurities which had no enzymatic activity. Peak II constituted the prorennin fraction, while a minor peak (III) represented active rennin. A minor peak between peaks I and II was inactive impurities. In all preparative experiments activity applied to the column as partially purified prorennin was completely recovered in the prorennin and rennin fractions (peaks II and III). The activated enzyme (peak III) amounted to only 0.1% of the total potential activity when chromatographed at 3 C, but tended to increase when chromatographed at higher temperatures. The purified prorennin fraction was never entirely free from active enzyme because some activation occurred during and after fractionation.

There was no evidence of chromatographic heterogeneity in the prorennin peak shown in Figure 1. Material represented by this peak was pooled, dialyzed and freeze dried. Some of the dry material was rechromatographed on a new preparative DEAE-cellulose column with an elution gradient from 0.1 to 0.3 M phosphate buffer at pH 5.85. Figure 2 shows the effluent curve for this experiment. Except for a minor peak representing activated enzyme, nothing emerged from the column but a single peak which represented the prorennin fraction. Again there was no evidence of heterogeneity.

Further evidence of homogeneity of the prorennin was provided by

Figure 1. Chromatography of partially purified prorennin on a DEAE-cellulose column (2.5 x 28 cm). 650 mg sample equilibrated with 0.05 M phosphate buffer (pH 5.95). Elution was stepwise as indicated. Flow rate: 36 ml/hr. Fraction size: 21.2 ml.

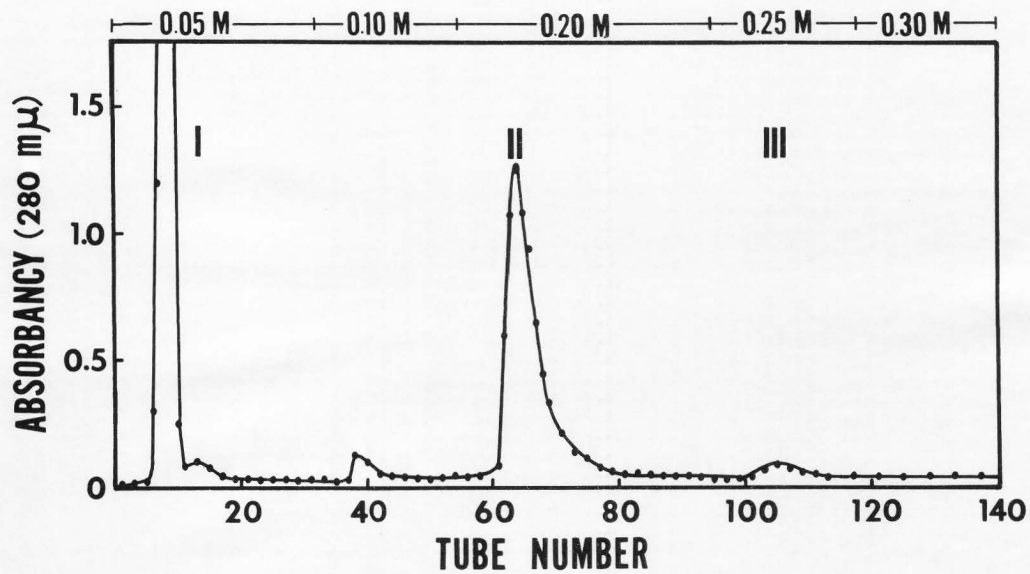
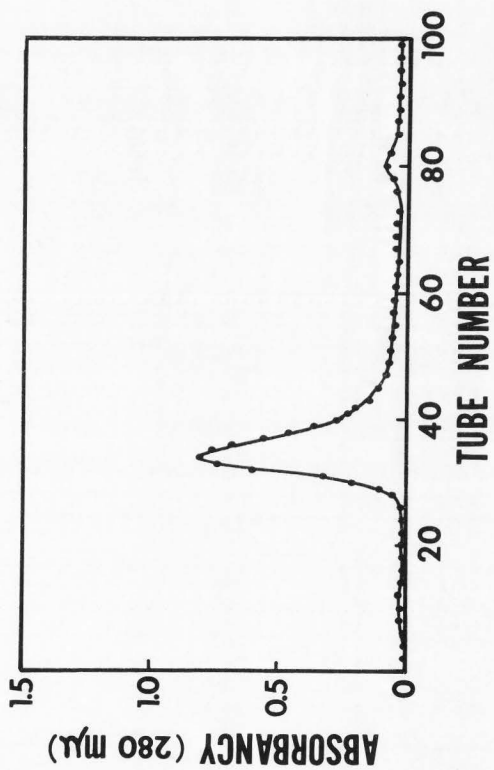


Figure 2. Rechromatography of prorennin by gradient elution on a DEAE-cellulose column (2.5 x 25 cm). Gradient formed by starting with 800 ml 0.1 M phosphate buffer at pH 5.85 and adding 800 ml 0.3 M phosphate buffer at pH 5.6 during chromatography. Flow rate: 25 ml/hr. Fraction size: 18 ml. Load: 130 mg.



results obtained from zone electrophoresis in starch-urea-gel. Figure 3 shows the results of this experiment. While crystalline rennin (D) was resolved into six components, prorennin (A, B, and C) showed but a single major band. The figure also shows a minor low mobility band in A and C on samples subjected to a single chromatographic purification. This represented an impurity not present in sample B which had been double chromatographed. The impurity bands in A and C were not visible to the naked eye on the starch-urea-gel, but they were picked up by the camera. Although these electrophoretic impurities were separated from prorennin by rechromatography (Figure 3), there was no measurable increase in specific activity. Hence, prorennin used in this study was run only once through the DEAE-cellulose column unless otherwise specified.

The effect of adding sulfur-containing compounds to the protein solvent, in addition to that added to the gel, for electrophoresis was examined since it was known to improve resolution of some proteins (29). Figure 4 illustrates the effect when rennin and prorennin samples were dissolved in 6 M urea in the presence and absence of 1% (v/v) 2-mercaptoethanol, and applied to starch-urea-gel containing 2-mercaptoethanol. Both these proteins (A and C) gave identical patterns whether or not 2-mercaptoethanol was added (B and D) to the sample. Therefore, 2-mercaptoethanol was used only in making the gel, since this gave as good resolution as when added to the sample.

Effect of urea on rennin and prorennin. The value of starch-urea-gel electrophoresis might be questioned as a technique for studying enzymes such as rennin because of the denaturing effect of urea. However, the excellent resolution

Figure 3. Starch-urea-gel electrophoresis patterns of: (A) and (C) Chromatographed prorennin, (B) Rechromatographed prorennin, and (D) Crystalline rennin. All samples were dissolved in 6 M urea at concentration of 1%. Electrophoresis was run for 4 1/2 hours at 25 C; 175 V at 12 ma.

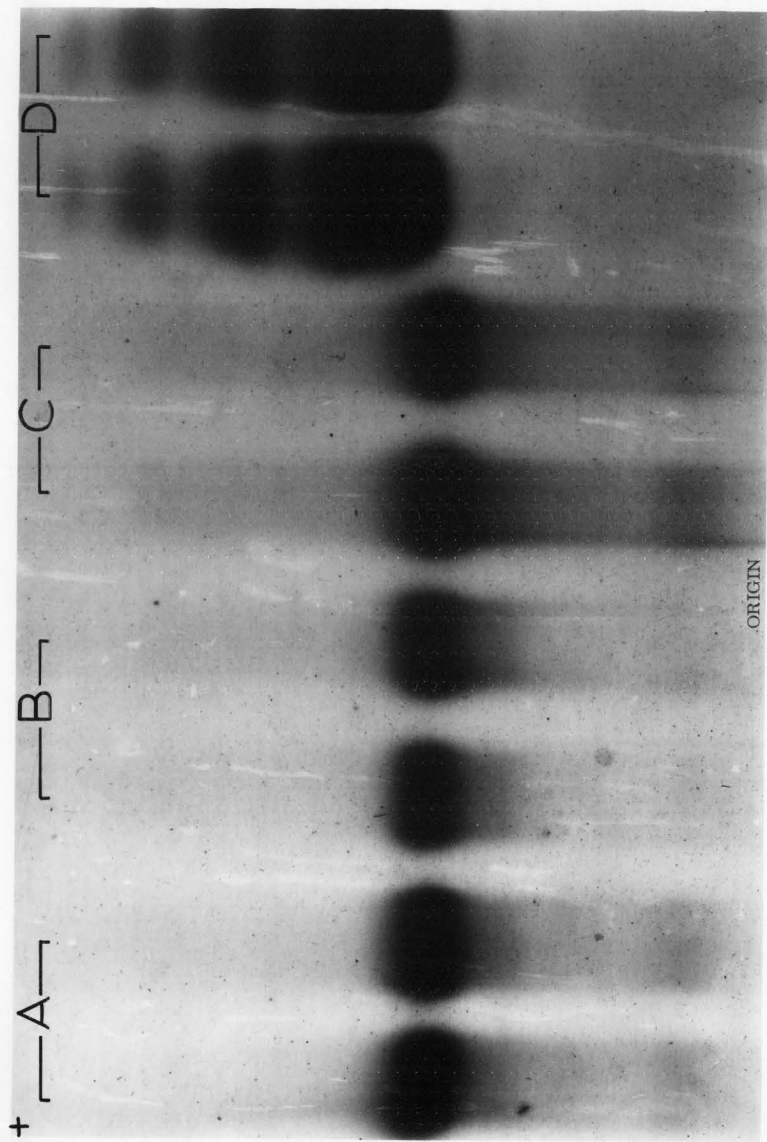
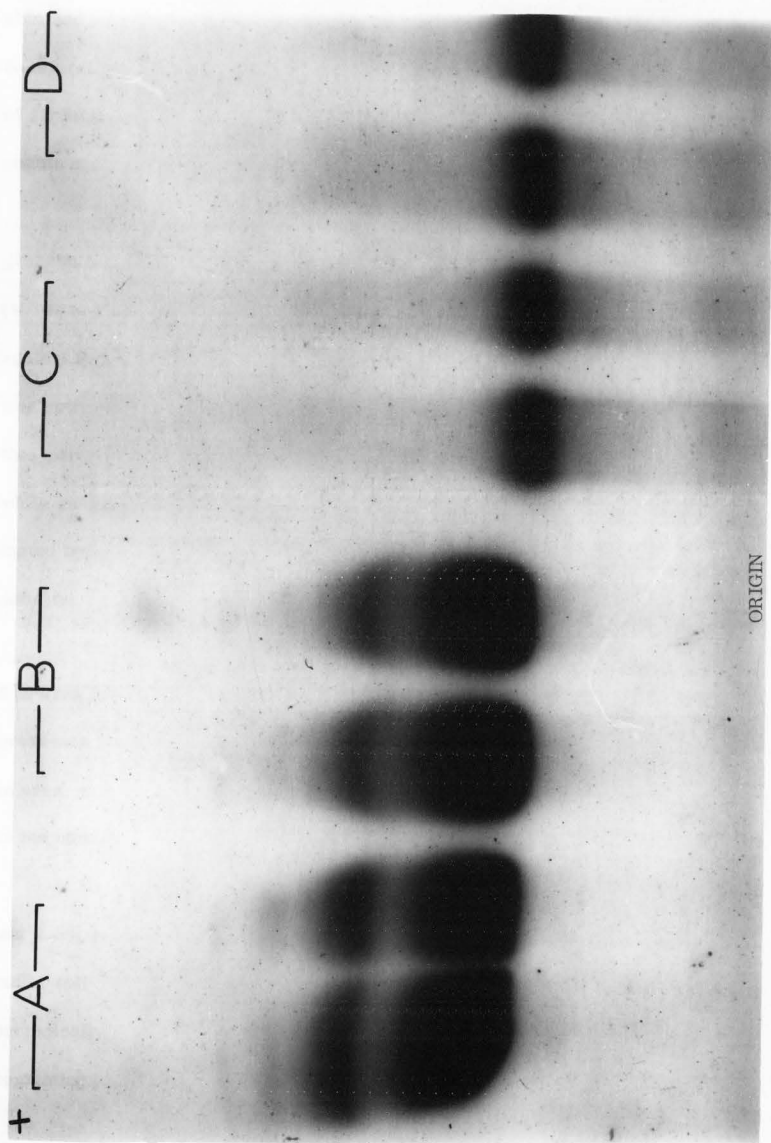


Figure 4. Starch-urea-gel electrophoresis patterns of: (A) Crystalline rennin dissolved in 6 M urea containing 1% (v/v) 2-mercaptoethanol, (B) Crystalline rennin dissolved in 6 M urea, (C) Chromatographed prorennin dissolved in 6 M urea containing 1% (v/v) 2-mercaptoethanol, and (D) Chromatographed prorennin dissolved in 6 M urea. Electrophoresis was run for 4 hours at 25 C; 175 V at 12 ma.



obtained with other proteins made it a tempting procedure. Even though urea denaturation does occur, the method can yield information about heterogeneity of structure and aggregation which can not be obtained from studies on native rennin and prorennin.

The effect of urea on enzyme activity was investigated. Samples of prorennin and rennin were dissolved in 6 M urea at a concentration of 1% (w/v), and left at room temperature for 6 hours after which they were dialyzed against 0.002 M cacodylate buffer at pH 6.0 for 6 hours. The prorennin sample was activated at pH 2.0 with dilute H_3PO_4 for 20 minutes. Activity tests were then performed on both samples. The "activated" prorennin showed no activity, while rennin contained 8% of its initial activity. When the rennin was tested before the urea was removed by dialysis, it contained 20% of its initial activity.

Crystalline rennin and purified prorennin that had been incubated in 6 M urea for different intervals, were electrophoretically assayed. Although prorennin patterns showed no significant change due to the prolonged incubation in urea, rennin patterns were changed significantly and indicated an increase in the number of components. This is shown in Figure 5.

Samples of crystalline rennin were dissolved at concentrations of 1% and 0.5% (w/v) respectively in 6 M urea and 6 M urea plus 0.05 M phosphate buffer (pH 5.8). The solutions were held at 25 C, and samples were withdrawn periodically for activity measurements. Results in Figure 6 show that solutions containing phosphate ions lost activity more rapidly than phosphate-free urea

Figure 5. Starch-urea-gel electrophoresis patterns of: purified prorennin and crystalline rennin incubated in 6 M urea for different intervals: (A, C, E, and G) Prorennin incubated for 0, 6, 12, and 24 hours respectively, (B, D, F, and H) Rennin incubated for 0, 6, 12, and 24 hours respectively. Electrophoresis was run for 4 1/2 hours at 25 C; 175 V at 12 ma.

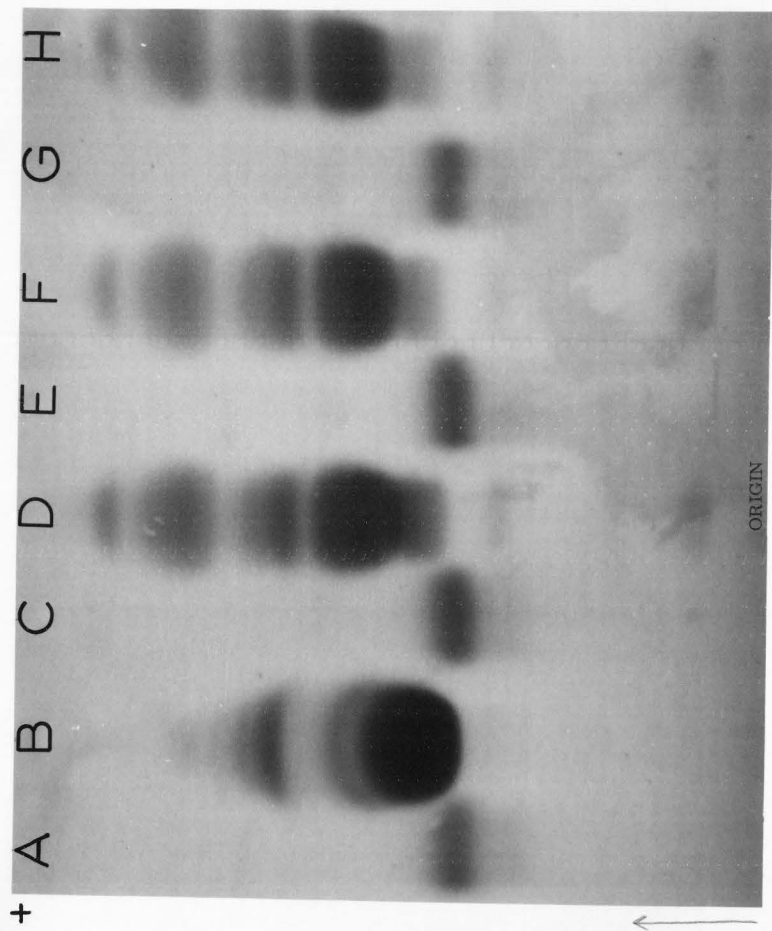
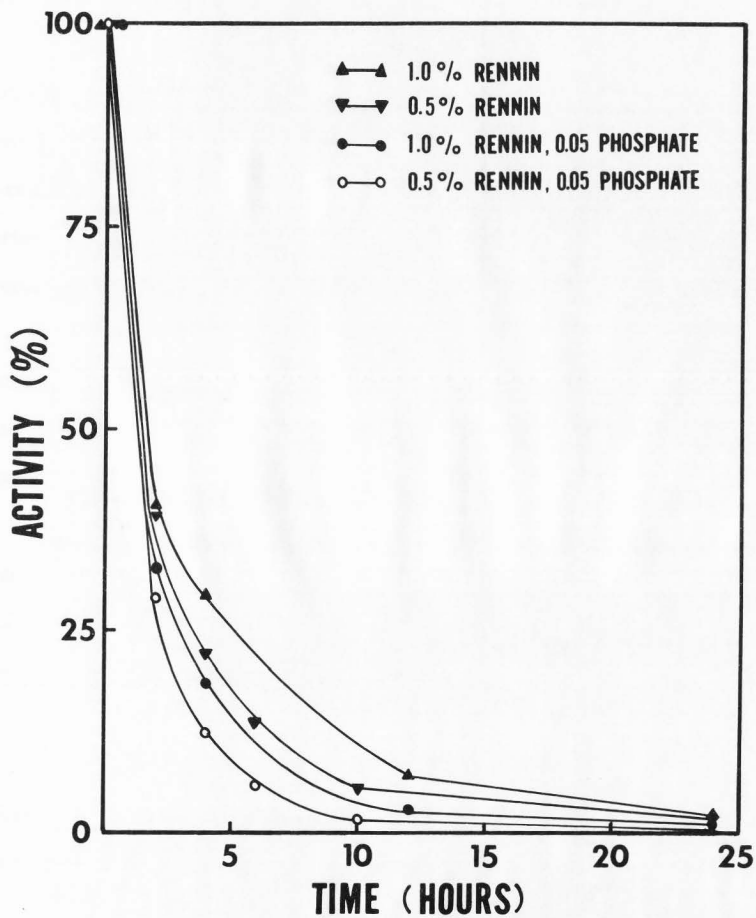


Figure 6. Effect of 6 M urea on rennin activity.

- ▲—▲ 1.0% rennin in 6 M urea.
- ▼—▼ 0.5% rennin in 6 M urea.
- 1.0% rennin in 6 M urea plus 0.05 M phosphate.
- 0.5% rennin in 6 M urea plus 0.05 M phosphate.






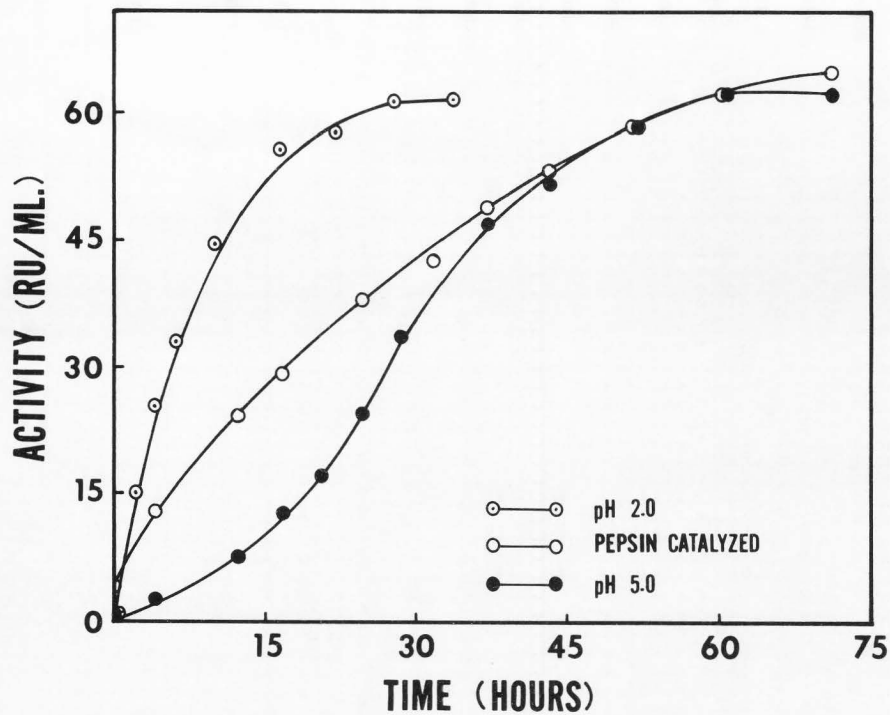
solutions. There was a more rapid loss of activity in 0.5% than in 1.0% rennin solutions. This was true whether or not the urea solutions contained added phosphate.

Effect of pH on activation of prorennin. Activation of prorennin was carried out at pH 2.0 and 5.0 in 0.02 M phosphate and 0.3 M acetate buffers respectively. The phosphate buffer was made to contain 60% glycerol (v/v) to reduce the rate of activation (37). A pepsin-catalyzed activation also was performed at pH 5.5 in 0.3 M citrate buffer (34). Activation mixtures were prepared by dissolving purified prorennin powder in the appropriate buffer at a concentration of 1.7 mg/ml. Pepsin in 0.3 M citrate buffer (pH 5.5), was added to the prorennin solution. Samples were withdrawn at appropriate intervals for activity measurements. A comparison of the activation of prorennin at different pH values is shown in Figure 7.

At pH 2.0, in the presence of glycerol, activation resembled a first-order reaction (37). At pH 5.0 the autocatalytic nature of activation was evident from the typical S-shaped curve characteristic of this reaction (22). A rather complex reaction occurred when pepsin catalyzed the activation. Maximum activity was reached in about 34 hours at pH 2.0 in phosphate buffer containing 60% (v/v) glycerol. At pH 5.0, maximum activity was attained in about 60 hours. At pH 5.5, in the presence of 4 milk-clotting units/ml of pepsin, the activation was complete after about 70 hr.

Resolution of activation mixtures. Crystalline rennin and chromatographically purified prorennin were chromatographed on DEAE-cellulose columns

Figure 7. Pepsin-catalyzed activation of prorennin compared with activation at pH 2.0 and 5.0. Activation at pH 2.0 in phosphate buffer containing 60% (v/v) glycerol  ; Activation at pH 5.0 in 0.3 M acetate buffer  ; Pepsin-catalyzed activation at pH 5.5 in 0.3 M citrate buffer .



under the same conditions used for resolution of activation mixtures. Elution was carried out with phosphate buffer with a linear gradient between 0.02 M (pH 6.0) and 0.4 M (pH 5.5). These chromatograms provided a basis of comparison for those obtained during chromatographic separation of activation mixtures. They also served to eliminate doubt that some fractions separated from activation mixtures were present in the original prorennin material.

Figure 8 shows the elution curve for purified prorennin which did not show evidence of impurities except for some activated enzyme. The prorennin appeared to be homogeneous by both absorbancy and activity curves. Unlike prorennin, crystalline rennin gave chromatographic evidence of heterogeneity as shown in Figure 9. Two rennin fractions, C and B (14), were observed along with a small fraction of inactive material (impurities) which was eluted earlier as shown in the figure. When rennin fractions were electrophoretically analyzed, it appeared that C-rennin consisted of the minor fractions (five), while B-rennin contained the major one. The front portion (the first 8 tubes) of B-rennin, however, showed some contamination from C-rennin and visa versa. This is shown in Figure 10.

Activation mixtures were prepared by dissolving purified prorennin in an appropriate buffer for activation. After activation was complete the mixtures were neutralized to $\text{pH } 6.0 \pm 0.1$ with saturated Na_2HPO_4 , dialyzed against 0.002 M cacodylate buffer (pH 6.0), and applied to an analytical DEAE-cellulose column (38 x 1.1 cm). Fractions (5-ml) were collected and their absorbancies measured at 280 m μ . Selected samples were assayed for rennin activity. Figures 11 and 12 show elution chromatograms for prorennin

Figure 8. Rechromatography of prorennin by gradient elution on a DEAE-cellulose column (38 x 1.1 cm). Linear elution gradient formed by starting with 0.02 M phosphate buffer at pH 6.0 and going to 0.4 M phosphate buffer at pH 5.5. Flow rate: 15 ml/hr. Fraction size: 5 ml. Load: 30 mg.

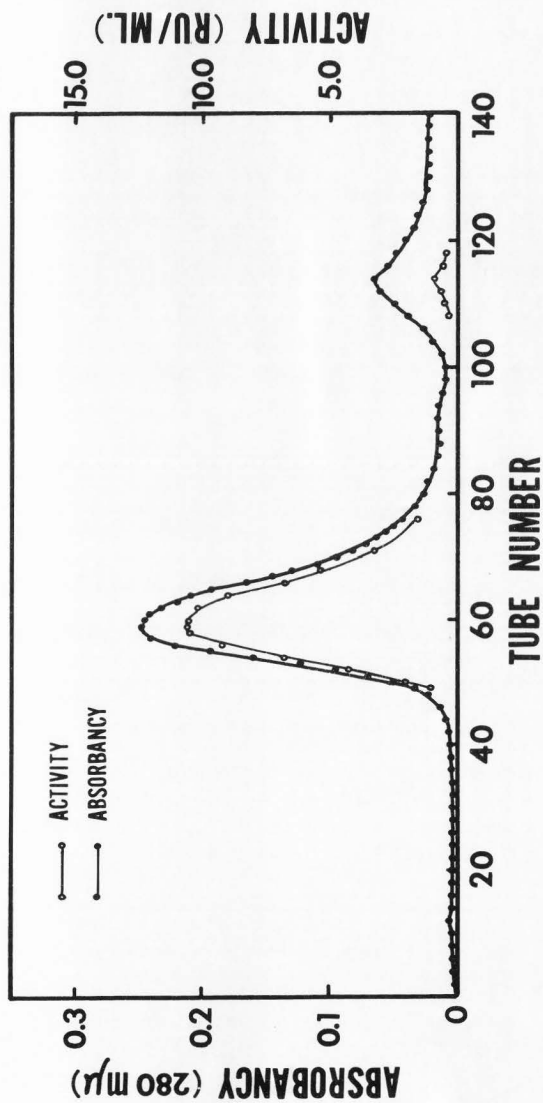


Figure 9. Chromatography of crystalline rennin by gradient elution on a DEAE-cellulose column (38 x 1.1 cm). A linear elution gradient formed by starting with 0.02 M phosphate buffer at pH 6.0 and going to 0.4 M phosphate buffer at pH 5.5. Flow rate: 20 ml/hr. Fraction size: 5 ml. Load: 33 mg.

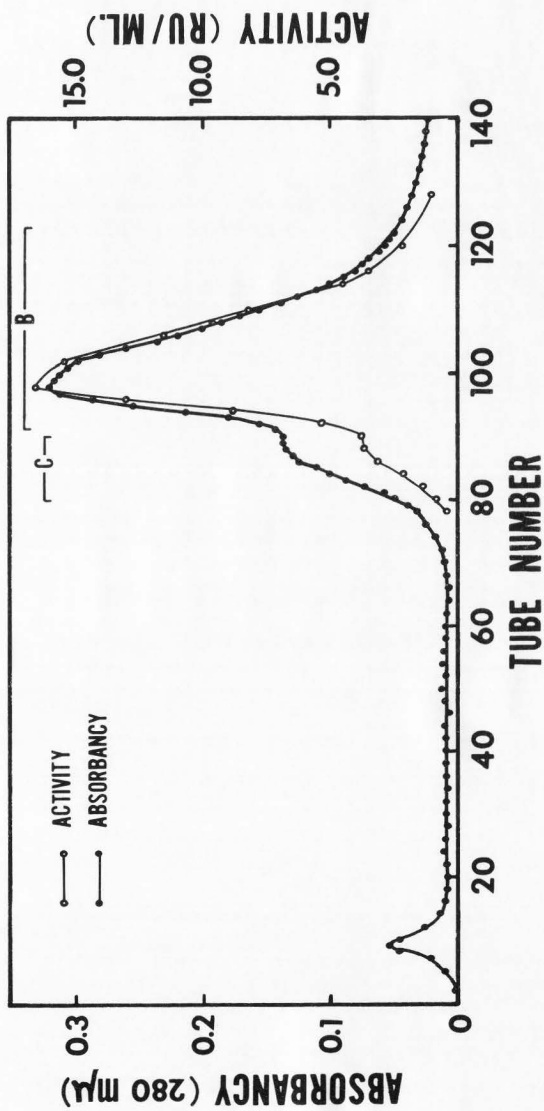


Figure 10. Starch-urea-gel electrophoresis of: (A and E) Whole crystalline rennin, (B) C-rennin, (C) The front portion of B-rennin (adjacent to C-rennin), (D) The second portion of B-rennin. Electrophoresis: 4 hours at 25 C; 175 V and 12 ma. Protein concentration: 1%.

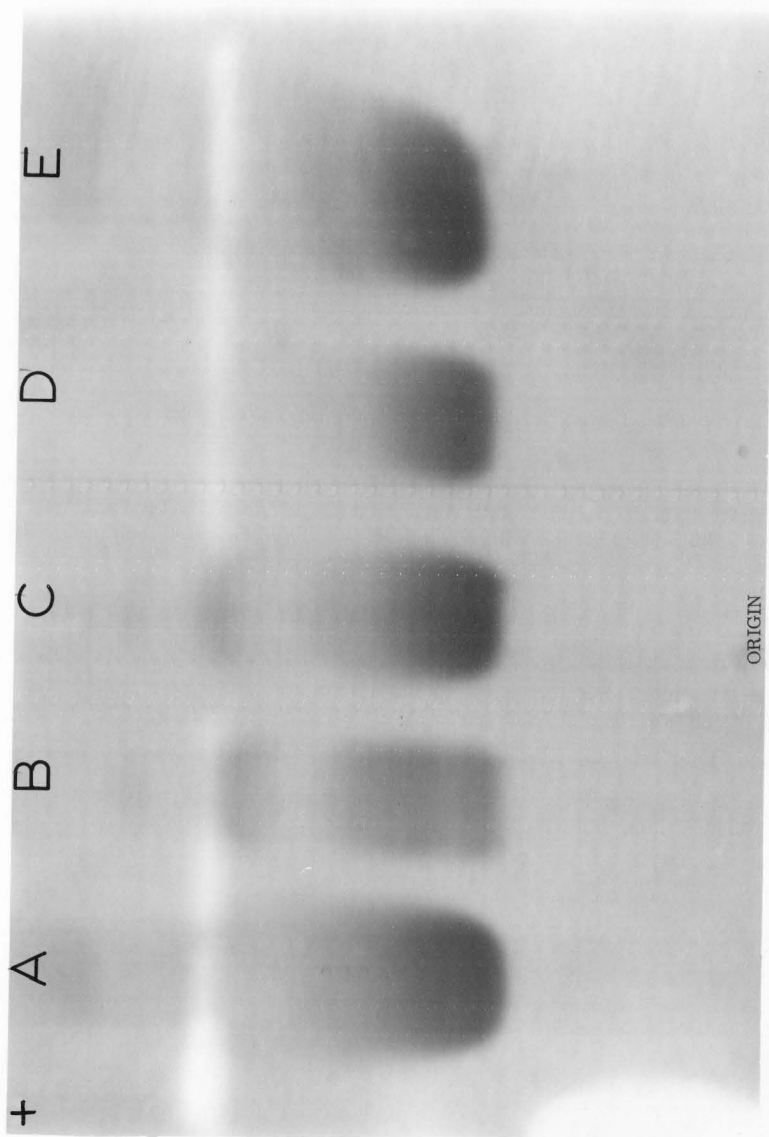


Figure 11. Chromatography of activation mixture (pH 2.0) by linear gradient elution on a DEAE-cellulose column (38 x 1.1 cm). Elution gradient from 0.02 M phosphate buffer at pH 6.0 to 0.4 M phosphate buffer at pH 5.5. Flow rate: 15 ml/hr. Fraction size: 5 ml. Load: 34 mg.

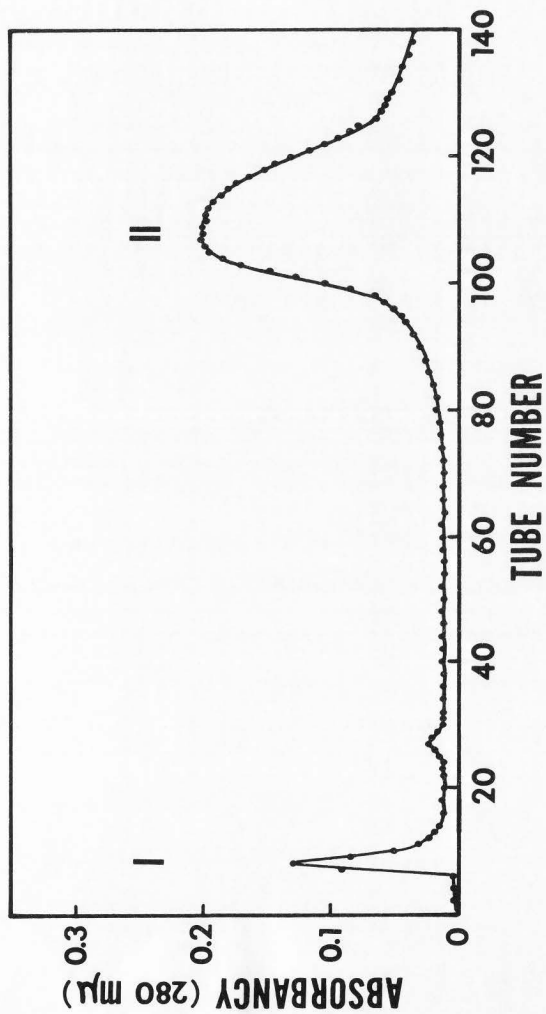
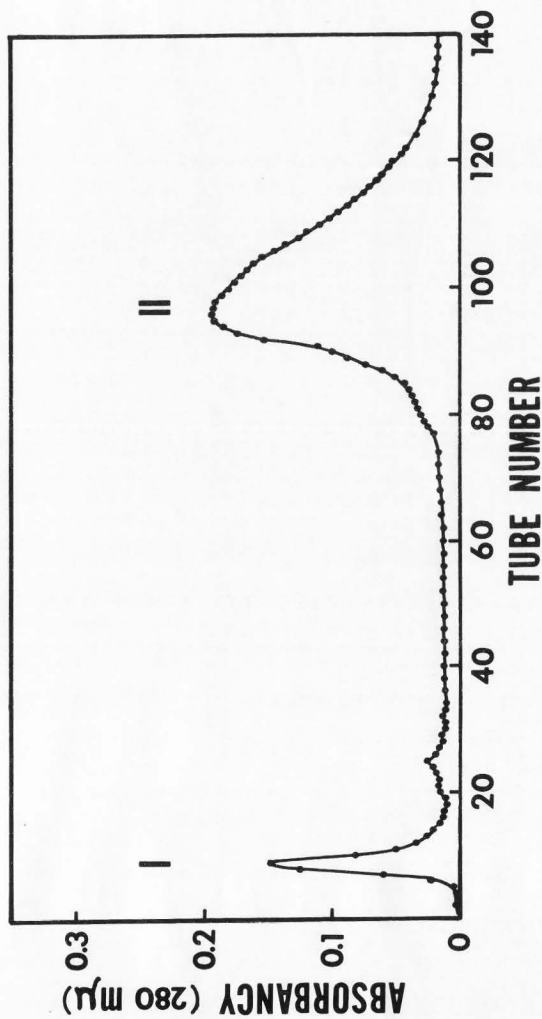


Figure 12. Chromatography of activation mixture (pH 5.0) by linear gradient elution on a DEAE-cellulose column (38 x 1.1 cm). Elution gradient from 0.02 M phosphate buffer at pH 6.0 to 0.4 M phosphate buffer at pH 5.5. Flow rate: 15 ml/hr. Fraction size: 5 ml Load: 34 mg.

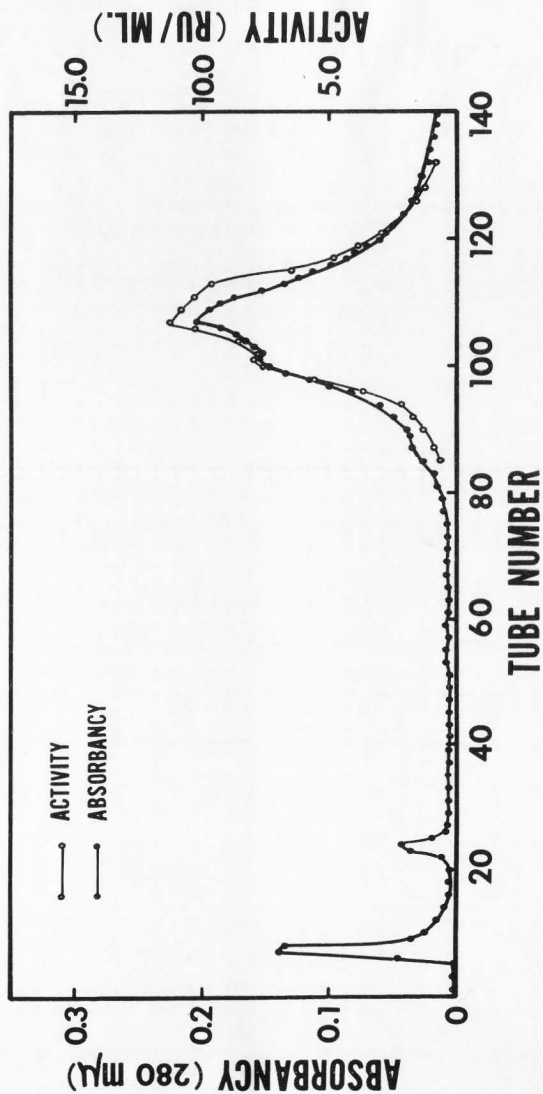


following activation at pH 2.0 and 5.0 respectively. In Figure 11 two major peaks are observed; the peptide (I) and the rennin (II). A minor peak adjacent to the main peptide peak is also visible. The highest concentration of rennin was eluted at an elution volume of about 540 ml. Fractionation of the mixture activated at pH 5.0 (Figure 12) gave a similar curve, but elution of the rennin fraction did not require as high a concentration of phosphate buffer. In this case the highest concentration of the enzyme came off the column at an elution volume of about 480 ml. Slight heterogeneity may also be noted in the chromatogram. It was difficult to detect any chromatographic differences between peptide fractions from the two activation mixtures.

To confirm elution differences observed between rennin from the two activation mixtures, samples of prorennin were activated at pH 2.0 and 5.0 respectively. After activation was complete, the activation mixtures were neutralized and dialyzed as described previously then freeze dried. The dried materials were mixed, dissolved in few milliliters equilibrating buffer and immediately applied to the DEAE-cellulose column. Fractionation of the mixture is illustrated in Figure 13. Two overlapped fractions are evident. The maximums of these fractions were eluted at about the same positions as for the individual activation mixtures. The peptide fractions were also observed as shown in the figure.

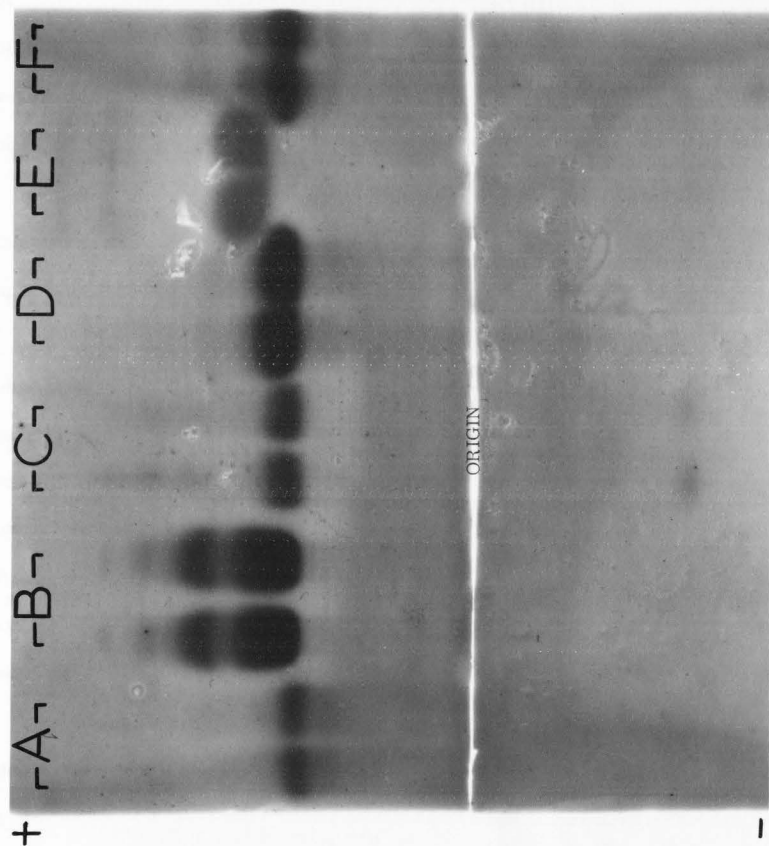
Electrophoretic resolution of activation mixtures. Activation of prorennin was carried out in the absence of glycerol at pH 2.0 and 5.0. Activation of a third sample was catalyzed by pepsin at pH 5.5 (34). When activation was

Figure 13. Chromatography of mixed activation mixtures (activated at pH 2.0 and 5.0) by gradient elution on a DEAE-cellulose column (38 x 1.1 cm). Elution gradient from 0.02 M phosphate buffer at pH 6.0 to 0.4 M phosphate buffer at pH 5.5. Flow rate: 15 ml/hr. Fraction size: 5 ml. Load: 35 mg.



complete (near completion for pepsin-catalyzed activation), the mixtures were neutralized to $\text{pH } 6.0 \pm 0.1$ with saturated Na_2HPO_4 , dialyzed against 0.002 M cacodylate buffer ($\text{pH } 6.0$), and lyophilized. A 1% solution of each dried activation mixture in 6 M urea was applied immediately to a starch-urea-gel slab for electrophoresis. Samples of prorennin, crystalline rennin, and pepsin were run simultaneously for identification and comparison. Electrophoresis was carried out for 4 1/2 hours at 175 V and 12 ma. After the run was completed the gel was stained with amido black. Figure 14 shows the starch-urea-gel electrophoretic pattern from this experiment. Prorennin (A) showed a single band while crystalline rennin, (B) was resolved into six components. The mixture activated at $\text{pH } 2.0$, (C) was resolved into five fractions; one major rennin component traces of three others, and a positively charged fragment which moved toward the negative electrode. When prorennin was activated at $\text{pH } 5.0$, (D) only one major band representing a single rennin component was observed. The positively charged component noted in the $\text{pH } 2.0$ activation mixture, was absent in the $\text{pH } 5.0$ activation mixture. Pepsin (E) gave a single electrophoretic band although a trace of heterogeneity may be noted. Electrophoretic patterns of the pepsin-catalyzed activation mixture (F) and the $\text{pH } 5.0$ activation mixture (D) were quite similar. Evidence of some prorennin and pepsin may be observed in pattern (F). A slight heterogeneity comparable to that of crystalline rennin was observed in all activation mixtures. It was noted that the main rennin component activated at $\text{pH } 5.0$ moved slightly ahead of that activated at $\text{pH } 2.0$. It was also observed that rennin activated at

Figure 14. Starch-urea-gel electrophoresis patterns of: (A) Prorennin, (B) Crystalline rennin, (C) pH 2.0 activation mixture, (D) pH 5.0 activation mixture, (E) Crystalline pepsin, and (F) Pepsin-catalyzed activation mixture. Electrophoresis was run for 4 1/2 hours at 25 C; 175 V at 12 ma.



pH 5.0 always gave darker bands upon staining than that activated at pH 2.0.

There was no electrophoretic evidence of the large inactive peptide fraction separated from prorennin during activation. This fraction, however, was readily separated from active rennin by column chromatography. Absence of these peptide fractions in the electrophoretic patterns was due to their inability to stain with amido black. This was confirmed when peptide materials separated by column chromatography were electrophoretically analyzed, and could not be detected in the starch gel.

It is noteworthy that the positively charged fraction which appeared in the electrophoretic pattern of the mixture activated at pH 2.0 became lighter upon continued destaining.

Wisse et al. (44) reported better resolution of protein components and sharper electrophoretic zones when protein samples were held in urea solutions prior to electrophoresis. Hence, a portion of the samples used in the previous experiment were left at room temperature for 4 1/2 hours before they were analyzed by electrophoresis. Figure 15 illustrates the effect of such treatment. All samples showed sharper bands. This was particularly true for crystalline rennin in which resolution was improved (B), and, for the pepsin-catalyzed activation mixture (F).

Amino acid analysis. Amino acid analysis was carried out as previously described. Samples of prorennin and the rennin prepared from it at pH 2.0 and 5.0 were analyzed. Table 2 shows the results of this analysis. As far as prorennin is concerned, the results are in good agreement with those reported

Figure 15. Starch-urea-gel electrophoresis patterns of: (A) Prorennin, (B) Crystalline rennin, (C) pH 2.0 activation mixture, (D) pH 5.0 activation mixture, (E) Crystalline pepsin, and (F) Pepsin-catalyzed activation mixture. Samples were held in urea 4 1/2 hours before electrophoresis for 4 1/2 hours at 25 C; 175 V at 12 ma.

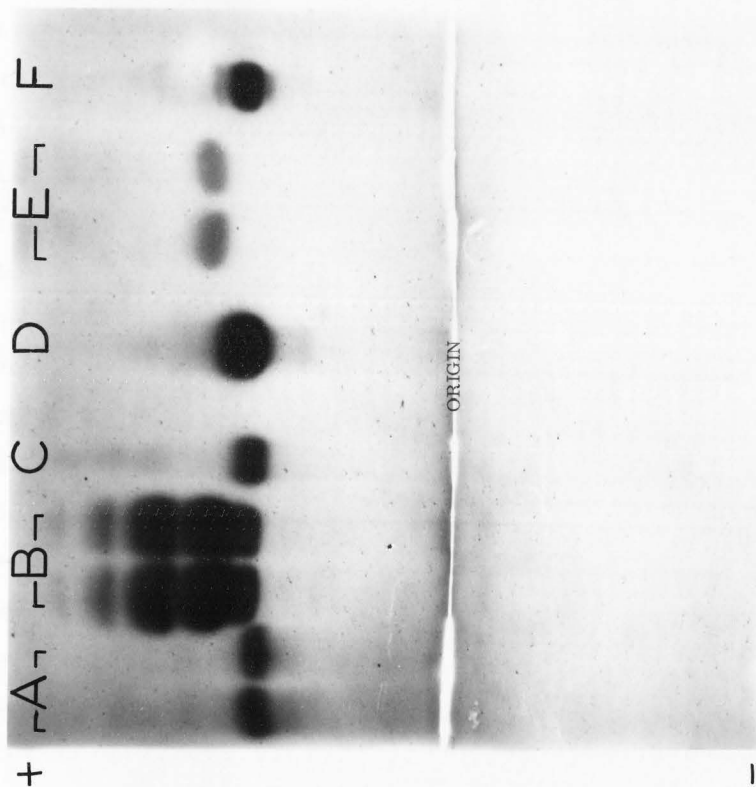


Table 2. Amino acid composition of prorennin, rennin activated at pH 2.0, and rennin activated at pH 5.0 expressed as moles/10⁵ grams protein^a

Amino acid	Prorennin	Rennin	
		pH 2.0	pH 5.0
Lysine	38.9	26.1	26.0
Histidine	14.5	13.9	13.2
Arginine	21.7	24.9	17.9
Asparatic	91.7	107.8	106.1
Threonine	56.8	64.7	63.0
Serine	71.3	80.1	77.7
Glutamic	102.8	97.6	98.8
Proline	42.7	32.8	43.1
Glycine	77.2	81.0	79.6
Alanine	48.5	43.4	38.3
Cystine 1/2	15.1	9.2	11.2
Valine	57.2	66.1	69.0
Methionine	15.5	7.1	8.9
Isoleucine	49.0	49.7	47.4
Leucine	69.3	59.7	65.0
Tyrosine	48.1	45.3	49.7
Phenylalanine	43.9	44.5	46.1

^a The nitrogen content reported by Foltmann (16) for prorennin and rennin (15.5 and 15.4% for prorennin and rennin respectively) were used to calculate the amino acid composition of these proteins.

by Foltman (17) for total prorennin or prorennin-B. Except for arginine and proline, the amino acid compositions of the two rennin samples are very close. It should be noted that the arginine content of Foltmann's (17) rennin is closer to that of the rennin activated at pH 5.0 than to that activated at pH 2.0.

DISCUSSION

Purified prorennin appeared to be homogeneous when subjected to chromatography on DEAE-cellulose and to electrophoresis on starch-urea-gel. This was contrary to the findings of Foltmann (15, 16), but in agreement with those of Bundy et al. (5) and Djurtoft et al. (7). Foltmann (18) suggested that the heterogeneity observed in his work might have stemmed from genetic variants that were not present in the prorennin used by Bundy et al. (5). However, no evidence was given to support the existence of genetic variants in prorennin. Foltmann (18) did not suggest a reason for the homogeneity reported by Djurtoft et al. (7) who used the same material he did.

When chromatographically purified prorennin solutions were concentrated by perfusion in dialysis casings prior to freeze drying there was about 5% loss in activity accompanied by slight chromatographic heterogeneity in the dried material. However, this heterogeneity was produced during perfusion and did not exist in the original material. A similar observation was made by Gellotte and Krantz (19) when they sustained some loss of activity during vacuum concentration of highly purified pepsin solutions.

There have been numerous reports (9, 14, 25) that crystalline rennin is a heterogeneous protein. These findings were verified by both column chromatography and zone electrophoresis. This, however, did not rule out the possibility that a homogeneous rennin might be obtained since an essentially

homogeneous rennin was observed by chromatography and electrophoretic analysis of freshly activated prorennin. Crystalline pepsin was long considered a heterogeneous protein until Rajagoplan et al. (32) showed that autolysis usually takes place during its preparation which requires prolonged treatment under acid conditions. They also showed that the homogeneity of pepsin depended critically upon the pH of activation. This is most probably the case with crystalline rennin, since all the crystalline rennin used in reported studies (includes the present one) were prepared from commercially activated rennet extract. Rennin has been shown to undergo autolysis under certain conditions (28).

It appears that a rennin fraction which corresponded to Foltmann's (14, 18) B-rennin produced a single electrophoretic component, but that several components were evident in the electrophoretic pattern of the fraction which corresponded to his C-rennin. When activity losses occurred in purified rennin there was always an increase in the C component. This raises the possibility that the C-fraction is really a partially fragmented form of rennin and may consist of several components distinguishable by starch-urea-gel electrophoresis. Since C-rennin has a lower specific activity than B-rennin it is quite probable that some of these components are inactive.

Although it was suggested that rennin probably had no helical structure (25), the secondary structure of prorennin has yet to be determined.

Proteins may be denatured by urea which competes for H-bonding sites in proteins and produces an unfolding of the protein chain(s) (43). It was demonstrated that rennin had more resistance toward urea denaturation than prorennin.

This suggested a fundamental difference in their secondary or (and) tertiary structures. Even if rennin did not have a helical secondary structure (25), intramolecular H-bonding must be important to its activity. The potential activity of prorennin appeared to be more easily destroyed by urea, perhaps because the H-bonding sites are even more easily destroyed. An optical rotatory dispersion study would help greatly to learn about the structures of these proteins.

It was obvious that a change in primary structure occurred when prorennin was activated because there was a liberation of peptide material. Even though exposure of prorennin to 6 M urea did not increase the number of fractions observed in starch-urea-gel electrophoresis, it completely and rapidly destroyed its ability to become activated. On the other hand exposure of crystalline rennin to 6 M urea destroyed the enzyme activity more slowly, and caused an increase in the number of electrophoretic components.

As previously reported (33, 34, 37) there were distinct differences between activation curves for rennin activated at pH 5.0, pH 2.0, and for pepsin-catalyzed activation at pH 5.5. There also appeared to be differences between the products resulting from these different conditions of activation. When rennin samples activated at pH 2.0 and 5.0 respectively were eluted from DEAE-cellulose by gradient elution, the rennin activated at pH 5.0 came off the column earlier than that activated at pH 2.0. This was verified by combining the activation mixtures and chromatographed them together where they were eluted as distinguishable components. The relative positions of these two components on the chromatogram was such that the rennin

activated at pH 5.0 corresponded to the main component in crystalline rennin or to Foltmann's (14, 18) B-rennin. The rennin activated at pH 2.0 was eluted more slowly. It was obvious that neither of these represented the fragmented fraction which corresponded to Foltmann's (14, 18) C-rennin. It appeared that the rennin activated at pH 2.0 was in a position on the chromatogram which corresponded to Foltmann's (14, 18) A-rennin. It is interesting to speculate whether these components were the same.

Starch-urea-gel electrophoresis of the two activation mixtures showed that the main rennin component from pH 5.0 activation always migrated slightly ahead of the corresponding component activated at pH 2.0. It also showed that activation of prorennin at pH 2.0 produced a fragment which migrated toward the cathode in starch-urea-gel electrophoresis. This fragment was never present in mixtures activated at pH 5.0.

Amino acid analysis of rennin activated at these two pH values revealed that rennin activated at pH 2.0 contained more arginine and less proline (25 compared to 18 moles/ 10^5 g protein and 33 compared to 43 moles/ 10^5 protein for arginine and proline respectively). The difference in arginine content could have accounted for the differences in electrophoretic mobilities and chromatographic elutions.

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